Mitochondrial DNA phylogeography and population history of the grey wolf *Canis lupus*

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Abstract

**The grey wolf (*Canis lupus*) and coyote (*C. latrans*) are highly mobile carnivores that disperse
over great distances in search of territories and mates. Previous genetic studies have shown
little geographical structure in either species. However, population genetic structure is
also influenced by past isolation events and population fluctuations during glacial periods.
In this study, control region sequence data from a worldwide sample of grey wolves and a
more limited sample of coyotes were analysed. The results suggest that fluctuating popula-
tion sizes during the late Pleistocene have left a genetic signature on levels of variation
in both species. Genealogical measures of nucleotide diversity suggest that historical popula-
tion sizes were much larger in both species and grey wolves were more numerous than
coyotes. Currently, about 300 000 wolves and 7 million coyotes exist. In grey wolves, genetic
diversity is greater than that predicted from census population size, reflecting recent histor-
ical population declines. By contrast, nucleotide diversity in coyotes is smaller than that
predicted by census population size, reflecting a recent population expansion following
the extirpation of wolves from much of North America. Both species show little partitioning
of haplotypes on continental or regional scales. However, a statistical parsimony analysis
indicates local genetic structure that suggests recent restricted gene flow.**

Keywords: census, coyote, demography, mtDNA, nucleotide diversity, population structure

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Introduction

The immediate ancestors of the grey wolf (*Canis lupus*)
and coyote (*C. latrans*) were the late Pleistocene Eurasian
species *C. etruscus* and the North American early Pleis-
tocene form *C. lepophagus*, respectively (Nowak 1979).
Grey wolves were once widely distributed throughout
Europe, Asia and North America, and occupied a wide
variety of habitats including the dry Arabian desert, the
xeric Mediterranean shrublands, the coniferous forests
of Siberia and the frozen tundra on Ellesmere island (Mech
1970). However, over the last few centuries the wolf has
been extirpated from most of its former range (Young
& Goldman 1944). The surviving populations are often
geographically and genetically isolated from each other
(Ginsberg & Macdonald 1990; Wayne *et al*. 1992).

The historical distribution of the coyote was restricted
to the plains and deserts of central North America (Gier
1975; Bekoff & Wells 1986). With the disappearance of
wolves and the modification of landscapes that followed
the westward expansion of pioneers, the geographical
range of coyotes expanded to include all the USA and
most of south and northwest Canada (Macdonald 1984).
These recent demographic events have left their signature
on the genetic structure of both species. Coyote mitochon-
drial DNA (mtDNA) haplotypes are not geographic-
ally structured and coyotes interbreed with wolves in

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places where the wolf population has dramatically decreased (Lehman & Wayne 1991; Wayne & Jenks 1991; Roy *et al.* 1994). North American wolves have a rather continuous distribution throughout Canada and Alaska, and, as with the coyotes, no well-defined phylogeographical structure is observed (Roy *et al.* 1994). However, in Europe and perhaps in Asia, wolf populations are genetically isolated and have reduced genetic variability, possibly as a result of recent population bottlenecks (Wayne *et al.* 1992; Randi *et al.* 1995; Ellegren *et al.* 1996).

In this study, the genetic variability and relationships of grey wolves throughout the world based on control region sequences were assessed. The genetic variability of wolves was compared with coyotes, which provided insights into the origin of both species and their Pleistocene diversification. Finally, genetic information on wolves was integrated to identify populations with low levels of genetic variation and to define evolutionary and management units for conservation (Moritz 1994).

Materials and methods

Samples

Control region sequences of 167 and 59 grey wolves were obtained from a previous study on the origin of genetic variability in dogs (Vilà *et al.* 1997) and from population studies of grey wolves (Ellegren *et al.* 1996; Taberlet *et al.* 1996; Tsuda *et al.* 1997; Pilgrim *et al.* 1998), respectively. New sequences were obtained from blood and tissue samples of 33 additional wild and captive wolves (Table 1). Wolves from areas where wolf–coyote hybridization is known to occur (Lehman *et al.* 1991) and which were found to carry coyote-like mtDNA sequences, have been excluded from the analysis. From the study of Ellegren *et al.* (1996), only wild Scandinavian wolves and wild-caught founders of the captive population were considered. In total, mtDNA sequences from 259 wolves from 30 localities worldwide were analysed (Table 1). The frequency of close relatives in the sample is probably low because samples were collected opportunistically over many years and over a wide area at each locality.

Tissue samples of 12 coyotes from different localities in North America were analysed (California, $n = 4$; Florida, $n = 1$; Louisiana, $n = 1$; Manitoba, $n = 1$; Michigan, $n = 1$; Texas, $n = 2$; Utah, $n = 1$; and Washington, $n = 1$). Sequences from faeces of Mexican ($n = 2$) and Minnesotan ($n = 1$) canids and a Texan red wolf (*Canis rufus*) which had sequences classified with those from coyotes, were also included. Finally, published sequences from two Montanan coyotes (Pilgrim *et al.* 1998) were included. One Ethiopian wolf (*C. simensis*), one golden jackal (*C. aureus*) and one black-backed jackal (*C. mesomelas*) were sequenced to be used as outgroups in the phylogenetic analysis.

DNA extraction and amplification

DNA was isolated using slight variations on phenol–chloroform extraction methods (Sambrook *et al.* 1989). For the coyote faeces, DNA was isolated following Höss & Pääbo (1992). Amplification of a 350 bp fragment of the control region I (Saccone *et al.* 1987) was performed via the polymerase chain reaction (PCR) using universal primers Thr-L 15926 5′-CAATTCCCCGGTCTTGTAAACC-3′ and DL-H 16340 5′-CCTGAAGTAGGAACCAGATG-3′ (modified from Kocher *et al.* (1989)). Extraction and no-template PCR controls were used in each amplification. Each PCR mixture contained approximately 100 ng of DNA, 25 pmol of each primer and 1 mM dNTP in a reaction buffer of 50 mM KCl, 2.5 mM MgCl$_2$, 10 mM Tris-HCl (pH 8.8), and 1.5 units of *Taq* DNA polymerase (Promega) in a total volume of 50 μL. Thirty-five cycles of amplification were performed in a programmable thermal cycler (Perkin-Elmer Cetus, Model 480). Each cycle consisted of denaturation at 94 °C for 60 s, annealing at 50 °C for 120 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 7 min. The PCR products were separated in a 1–2% Nusieve (FMC Corp.) agarose gel in TAE buffer. After staining with ethidium bromide, the appropriate band was excised, the DNA extracted using the Geneclean (BIO 101) or Ultra Clean 15 (Mo Bio Labs) kits, speed-vacuum dried, and eluted in 11–13 μL double-distilled H$_2$O.

DNA sequencing

Direct sequencing of double-stranded DNA (Sanger *et al.* 1977) was carried out using modifications of dimethylsulphoxide (DMSO)-based protocols (Winship 1989) and the Sequenase version 2.0 kit (US Biochemicals). The sequencing reaction products were separated by electrophoresis in a 6% polyacrylamide gel for 3 h at 55 W in a Stratagene Base Ace Sequencing apparatus. Sequence autorads were scored on an IBI gel reader, and entered into the MACVECTOR computer program (IBI-Kodak). Some samples were sequenced using dye terminator cycle sequencing chemistry on an ABI 377 instrument (Perkin-Elmer).

Sequences were aligned first by eye, then by using CLUSTAL V (Higgins *et al.* 1992), and rechecked by eye. Although over 350 bp of sequence information was obtained, only a fragment of 230–231 bp for the wolves and about 226 bp for coyotes, were considered consistent with all the studies in Table 1. This reduction in sequence size does not represent a significant loss of information, as the available information shows that only two variable positions for wolves are excluded, one of them representing a change that is present in only one sequence. Finally, one area of uncertain alignment, including a 19–20 bp segment in wolves and 11–14 bp in coyotes, was also excluded from the analysis for interspecific comparisons.

Table 1 Distribution of wolf haplotypes at each sampled location. The number of samples, different haplotypes and unique haplotypes are indicated for each column (population)

Haplotype	Portugal	Spain	France	Italy	Romania	Bulg.	Croatia	Yugos.	Greece	Poland	Sweden	Finland	Estonia	Russia	Turkey	Israel	S.Arabia	Iran	Afghan.	India	China	Mongol.	Alaska	Yukon	NWT	Alb.	Mont.	Minn.	Labra.	Mexico
lu-1	18	56																												
lu-2		1																												
lu-3	1						5		1		1			1*	2															
lu-4		27								1†																				
lu-5			7†	12+9†																										
lu-6					2+1†	1			1																					
lu-7					1						1*			1																
lu-8						1															1									
lu-9							1																							
lu-10								4‡	4																					
lu-34								3‡																						
lu-11									1																					
lu-12											1+14*	2	1+1*	1																
lu-13											1*			1																
lu-14																16														
lu-15																	2			1										
lu-16																	2													
lu-17																	1				1	1†+1‡								
lu-18																	1													
lu-19																		5												
lu-20																		1												
lu-21																			3											
lu-27																			5‡											
lu-22																					1									
lu-23																					1									
lu-24																						2‡								
lu-25																						2‡								
lu-26																						2‡								
lu-28																							3					1		
lu-29																								1						
lu-30																								1						
lu-31																								1						
lu-32																									3	1	1+3§		3	
lu-33																														6
n	19	84	7	21	4	2	6	7	7	1	18	2	2	4	2	16	7	6	8	1	3	8	3	3	3	1	4	1	3	6
Different haplotypes	2	3	1	1	2	0	2	2	4	1	4	1	1	4	1	1	5	2	2	1	3	4	1	3	1	1	1	1	1	1
Uniques	0	1	0	0	0	0	1	1	2	0	0	0	0	0	0	0	1	2	2	0	2	3	0	3	0	0	0	0	0	1

*Sequences from Ellegren *et al*. (1996); †sequences from Taberlet *et al*. (1996); ‡sequences from Tsuda *et al*. (1997); §sequences from Pilgrim *et al*. (1998).

Bulg., Bulgaria; Yugos., Yugoslavia; S. Arabia, Saudi Arabia; Afghan., Afghanistan; Mongol., Mongolia; NWT, Northwest Territories (Canada); Alb., Alberta (Canada); Mont., Montana (USA); Minn., Minnesota (USA); Labra., Labrador (Canada).

Relationship of mtDNA control region sequences

Nucleotide diversity, π (Nei & Li 1979), and its standard deviation (SD) for wolf and coyote haplotypes were estimated using the program DNASP (Rozas & Rozas 1997). For mtDNA data, the parameter θ equals $N\mu$, where N is the female effective population size and μ is the mutation rate per site per generation. An estimate of the effective number of females can be derived from this relationship if θ is estimated and a mutation rate is assumed. Tajima (1983) showed that $E(\theta) = \pi$, and we will denote this estimate as $\hat{\theta}_T$. However, this estimator does not use genealogical information, and therefore is not efficient (Felsenstein 1992). Consequently, a maximum likelihood estimator of θ denoted $\hat{\theta}_F$, that utilizes genealogical information and allows for variable population size was also used (Kuhner *et al.* 1995). To estimate $\hat{\theta}_F$ the computer program FLUCTUATE 1.3 was used (Kuhner *et al.* 1998).

To select the model of DNA substitution that best fitted the data, a hierarchical likelihood ratio test approach implemented in the program MODELTEST 1.03 was used (Posada & Crandall 1998). The model selected was the Hasegawa *et al.* (1985) model of substitution with rate heterogeneity (HKY + Γ). Using this model, the transition/transversion ratio (ti/tv) and gamma shape parameter (α) were 15.47 and 0.317 for the combined data set; 41.85 and 0.006 for coyotes; and 12.37 and < 0.001 for wolves. The phylogenetic relationships between haplotypes were reconstructed using the neighbour-joining method (Saitou & Nei 1987) under the HKY + Γ model of evolution with the parameter estimates given above. Confidence in estimated relationships was determined using the bootstrap approach (Felsenstein 1985). Bootstrap values were obtained through 1000 replicates incorporating the same model as above. Bootstrap analysis and phylogeny reconstruction were performed using PAUP* version 4.01b (Swofford 1998). The rates of evolution in coyote and wolf sequences were compared using Tajima's (1993) test.

The genetic similarity between populations may be due to ongoing gene flow or reflect recent colonization (Crandall & Templeton 1993). A statistical parsimony approach (Templeton *et al.* 1992) was used to construct a network to separate population history from population structure. Parsimonious ($P_j \geq 0.95$) connections were statistically justified for haplotypes that differed by up to six mutational differences (Templeton *et al.* 1992). A matrix of absolute pairwise differences was calculated considering gaps as a fifth state (program by D. Posada, available on request). This matrix was used to construct the statistical parsimony cladogram. Haplotypes were nested to better visualize higher-order patterns of association (Templeton & Sing 1993). Networks may more effectively portray the relationships among sequences for populations in which many sequences may be derived from the same ancestral genotype. Geographical association was tested for, treating each sample location as a categorical variable (Hudson *et al.* 1992). A permutational contingency analysis of the categorical variation was performed using the Roff & Bentzen (1989) algorithm for assessing the significance of the test statistic (program by D. Posada, available on request).

Regional patterns of geographical subdivision, gene flow and effective population sizes

The average sequence divergence between wolf populations was used to construct a neighbour-joining tree. One hundred bootstrap sequence data sets were similarly analysed to study the support of the neighbour-joining tree using the software PHYLIP 3.57c (Felsenstein 1989). This tree provided guidance in testing the significance of geographical population genetic units in an analysis of molecular variance (AMOVA) approach (Excoffier *et al.* 1992). AMOVA is a hierarchical analysis analogous to analysis of variance (ANOVA) in which the correlations among genotype distances at various hierarchical levels are used as F-statistic analogues, designated as Φ-statistics. Φ_{ST} is the correlation of random genotypes within a population relative to that from the whole species and is analogous to F_{ST} of Wright (1951), Φ_{CT} is the correlation of random genotypes within a group of populations relative to that drawn from the entire species and measures the proportion of genetic variation among groupings of populations, and lastly Φ_{SC} is the correlation of random genotypes within populations relative to that within a regional grouping of populations and measures the proportion of variation among populations within a region. The significance of these F-statistic analogues is evaluated by random permutations of sequences among populations. We experimented with various groupings of populations suggested by the analysis of DNA sequence and population trees (see above) and those suggested by taxonomy and geographical isolation. The groupings that maximized values of Φ_{CT} and were significantly different from random distributions of individuals were assumed to be the most probable geographical subdivisions.

Gene flow within and among regions was expressed as the number of female migrants per generation, N_m, where N is the female effective population size and m is the female migration rate. N_m was approximated by the expression $F_{ST} = 1/(1 + 2N_m)$ (Wright 1951; Slatkin 1987, 1993; Baker *et al.* 1994). Pairwise estimates of Φ_{ST} were used as surrogates for F_{ST} among regional groupings of populations and migration rates were calculated (Stanley *et al.* 1996). AMOVA, pairwise Φ_{ST} and N_m values, as well as the nucleotide diversity for each population, were calculated using ARLEQUIN 1.1 (Schneider *et al.* 1997). Maximum likelihood estimates of N_m were also obtained

using the program MIGRATE 0.6 (Beerli & Felsenstein submitted). This method uses a coalescent theory approach to estimate past migration rates between populations assuming a migration matrix model with asymmetric migration rates and different subpopulation sizes. The significance of the differences in nucleotide diversity between populations was assessed with a Kruskal–Wallis test (Sokal & Rohlf 1981).

Following Slatkin (1993), differentiation by distance was assessed by plotting pairwise $\log(N_m)$ values against log geographical distance. The significance of the association was determined by applying Mantel's permutation test (Mantel 1967). A significant association between N_m and distance indicates genetic structuring in populations and that dispersal of individuals is limited (Slatkin 1993).

Results

Sequence divergence in coyotes and wolves

Haplotype diversity was much greater in coyotes than in wolves. Thirty-four different mtDNA haplotypes were found in 259 wolves and 15 in 17 coyotes. Wolf sequences differed by one to 12 substitutions and had indels at two positions. Coyote sequences differed by one to 14 substitutions and had indels in seven positions. The nucleotide diversity (π or $\hat{\theta}_T$) among haplotypes in wolves, 0.026 (SD = 0.014), was significantly less than the value of 0.046 (SD = 0.025) in coyotes (Student's *t*-test, $P < 0.05$). By contrast, the maximum likelihood estimates of theta ($\hat{\theta}_F$) were considerably larger in both species, and the value of 0.744 (SD = 0.133) in wolves was significantly larger than the value of 0.373 (SD = 0.103) in coyotes (Student's *t*-test, $P < 0.05$).

The average within-species sequence divergence, corrected for multiple hits using the HKY mutation model ($\alpha = 0.317$), and with a transition/transversion ratio of 15.47, was 2.9% (standard error (SE) = 0.05, range: 0–7.4%) and 4.2% (SE = 0.20, range: 0.5–8.3%) for wolves and coyotes, respectively. These divergence estimates were significantly different (Student's *t*-test, $P < 0.001$). The average sequence divergence between wolves and coyotes was 13.1% (SE = 0.09, range: 8.0–19.2%).

The larger divergence among coyote sequences relative to that among grey wolves was also indicated by the longer terminal branch lengths of coyotes in a neighbour-joining tree (Fig. 1). In pairwise comparisons of wolf and coyote branch lengths using three outgroup species (Ethiopian wolf, golden jackal and black-backed jackal), we failed to find any evidence for substitution rate variation between species (Tajima (1993) test, $P > 0.05$). Consequently, these results suggest that coyote mitochondrial control region sequences diverged at a more ancient time than did sequences in grey wolves.

The distribution of the number of pairwise substitutions between haplotypes also differed between the two species (Fig. 2). Wolves had a strongly unimodal distribution with a modal value of five (mean: 5.27, SE = 0.09). By contrast, in coyotes, a distinct mode was not apparent, the distribution was ragged and values between four and 11 substitutions were equally common. The mean number of pairwise differences in coyotes, 7.35 (SE = 0.31), was significantly higher than that for wolves (Mann–Whitney *U*-test, $P < 0.001$). Although the mismatch distribution in wolves did not show significant kurtosis ($g_2 = -0.196$; Student's *t*-test, $P > 0.05$; Sokal & Rohlf 1981), the distribution was significantly platykurtic for coyotes, it was more clumped, broader and modal values were not well defined ($g_2 = -0.930$; Student's *t*-test, $P < 0.05$). The distribution of the pairwise distances between wolves and coyotes was clearly unimodal, with the modal values of 19 and 20 substitutions (mean: 18.79, SE = 0.10; Fig. 2c).

Phylogeography

Control region sequences of wolves were often restricted to a single locality or shared only between neighbouring localities (Table 1, e.g. lu-1, lu-2, lu-5, lu-6). However, a few haplotypes had a much wider distribution. The extreme example is lu-3, which was found in wolves from Portugal, Croatia, Greece, Sweden, European Russia and Turkey. Haplotype lu-8 was found in Bulgaria and Saudi Arabia; and lu-32 was found in the Northwestern Territories, Alberta, Montana and Labrador. In coyotes, only three haplotypes were found in more than one individual, and in each case, individuals with identical haplotypes were from the same locality.

Only 16 parsimony-informative sites were found for wolves and for coyotes. The small number of parsimony-informative sites, together with the high number of haplotypes (34 for wolves and 15 for coyotes) prevented full resolution of phylogenetic relationships using maximum likelihood and parsimony methods. The statistical parsimony technique, which is more powerful when the sequences differ in a few sites (Crandall & Templeton 1996), was used to understand relationships among genotypes and their correspondence with geographical distribution (Fig. 3; Crandall & Templeton 1993).

In general, Old World haplotypes from geographically neighbouring areas were linked by one or two substitutions in the statistical parsimony network (e.g. lu-1 and lu-2 from the Iberian Peninsula; lu-5, lu-6 and lu-34 from France, Italy, Romania, Bulgaria, Yugoslavia and Greece) (Fig. 3). However, overall, there was no clear geographical pattern in the distribution of haplotypes. Sequences from North American wolves clustered in three groups. One contained the three haplotypes found in Yukon, which were characterized by a unique insertion. Another
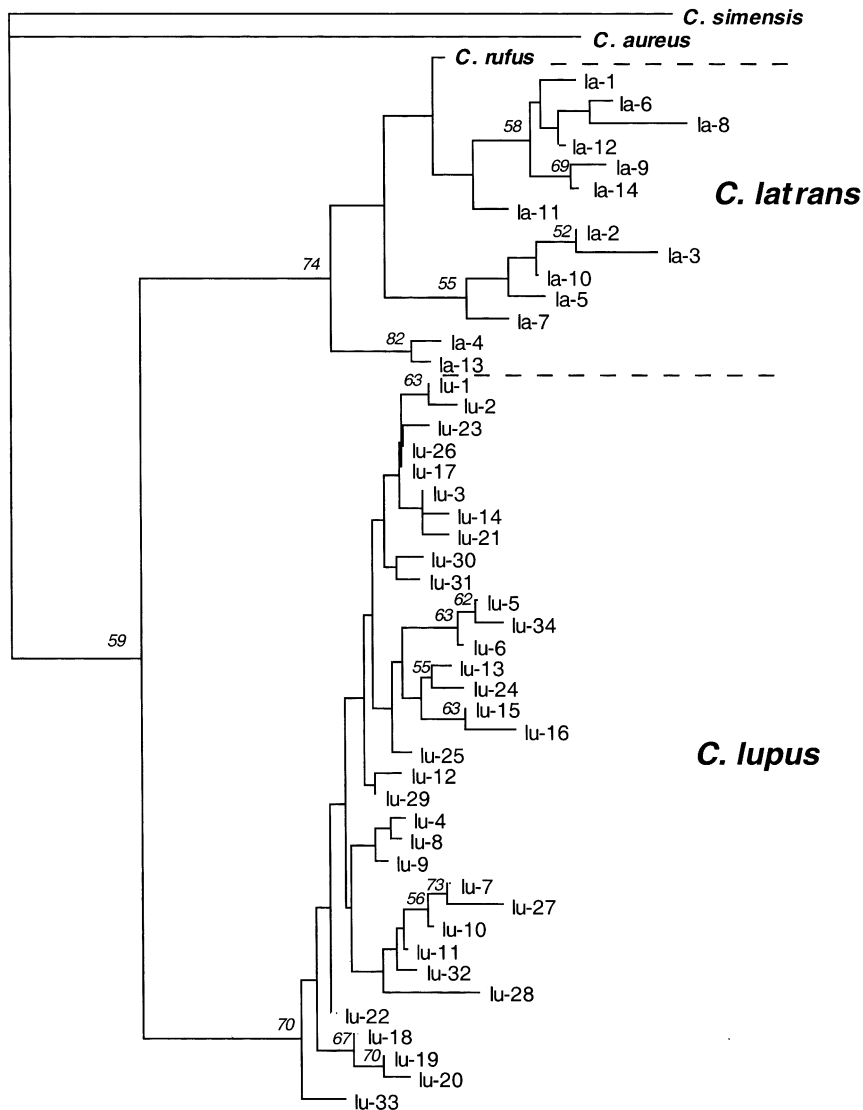
Fig. 1 Neighbour-joining tree based on the HKY model of sequence divergence with a gamma shape parameter of $\alpha = 0.317$ and a transition/transversion ratio of 15.47. Bootstrap support is indicated at nodes if found in more than 50% of 1000 bootstrap trees.

group contained the most widely distributed haplotypes, lu-28 and lu-32. Finally, the haplotype found only in Mexican wolves (lu-33) defined a distinct lineage, minimally five substitutions and one indel different from other North American grey wolf sequences. North American populations appear to have greater continuity and less subdivision than their Old World counterparts. The same sequence (lu-32) was found over a wide geographical range, and lu-28 was found both in Alaska and Minnesota. However, the sequences from Yukon formed a distinct group (lu-29, lu-30, lu-31).

Using the algorithm of Templeton *et al.* (1992), all control region haplotypes could be connected in a single network (Fig. 3). When changes were mapped in the cladogram the amount of parallelisms and reversals was high. At the 25 variable sites, 73 mutational steps were observed in the cladogram, resulting in an average of

nearly three changes per site. This high amount of homoplasy was also indicated by the extremely low value of the gamma shape parameter ($\alpha < 0.001$, see Materials and methods) which indicated a skewed distribution with the majority of changes occurring at a few sites. As most of the changes were transitions (ti/tv = 12.37), multiple hits in a single position will very often not be noticed as they would represent reversal to the ancestral condition. Consequently, the cladogram was complex and had loops connecting three or more haplotypes. Because this complexity makes the nesting procedure ambiguous, we did not perform a nested analysis (Templeton & Sing 1993) and only indicated a subset of the possible nested groups.

Some haplotypes from geographically neighbouring areas formed part of one-step clades (e.g. lu-1 and lu-2 from the Iberian Peninsula; lu-19 and lu-20 from Iran; lu-5 and lu-34 from France, Italy and Yugoslavia), but other
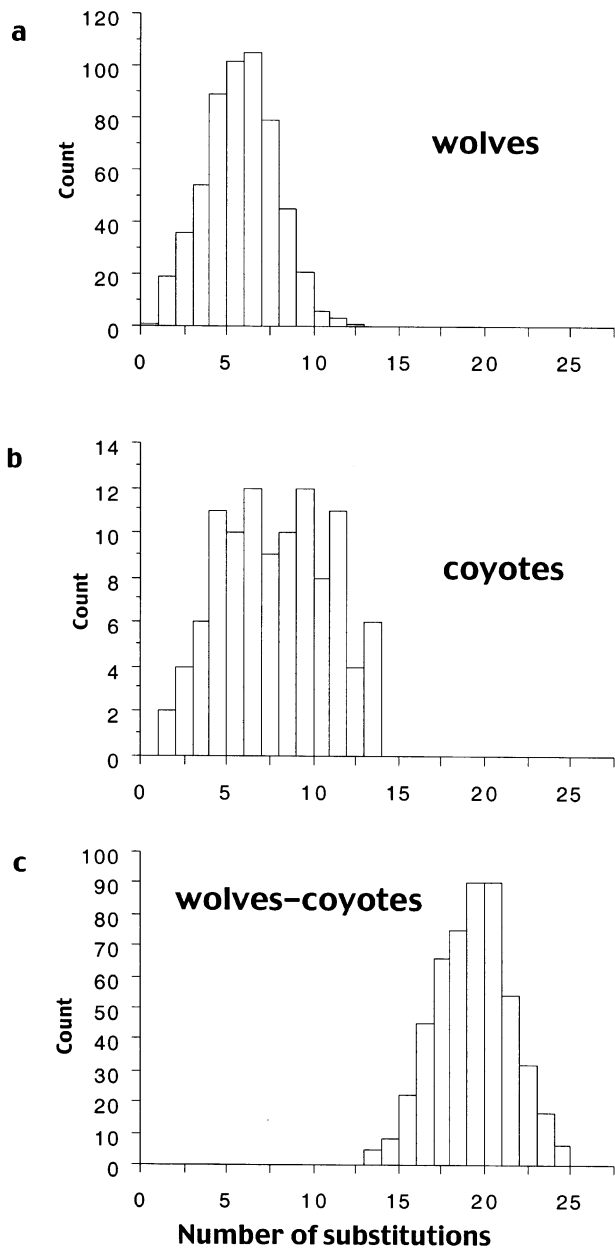
Fig. 2 Distribution of pairwise distances (measured as number of substitutions) (a) between wolf haplotypes, (b) between coyote haplotypes, (c) between wolf and coyote haplotypes. A region of difficult alignment between species has been excluded in all comparisons (see Materials and methods).

one-step clades included haplotypes from very distant localities (e.g. lu-12 and lu-29, from Northern Europe and Yukon). A permutation categorical contingency analysis of the whole cladogram rejected the null hypothesis of no association with geographical location ($P < 0.001$). Therefore, the distribution of lineages was not randomized with respect to geography. However, overall, there was no clear geographical pattern in the distribution of haplo-

types. Even at the continental level, haplotypes found in Europe, Asia and America were not grouped in exclusive clusters. Most of the Asian haplotypes appeared as interior nodes (Fig. 3) whereas the European and American haplotypes appeared in terminal positions. Haplotypes located at the tips of the cladogram tended to have restricted geographical distributions whereas ubiquitous, and presumably ancestral haplotypes, were on interior nodes (e.g. lu-3, lu-7, lu-8, lu-12, lu-17, lu-32).

Population diversity and relationships

Nucleotide diversity differed significantly among grey wolf populations (Tables 1 and 2; Kruskal–Wallis test, $H = 456.90$, d.f. = 13, $P < 0.001$). This result does not reflect sample size differences alone because nucleotide diversity did not increase with sample size as expected. Well-sampled European populations had lower nucleotide diversity and fewer divergent haplotypes (Portugal, Spain, Italy, Sweden or Israel) than populations in which only a few wolves had been sampled (e.g. Yugoslavia, Greece, Saudi Arabia, Afghanistan or Mongolia).

The general structure of the neighbour-joining population tree does not support higher-order groupings according to geographical proximity (Fig. 4). None of the resulting nodes was supported in 50% or more of 100 bootstrap trees. Some neighbouring populations were located on the same branches (e.g. Portugal and Spain; Italy, France and Romania; most North American populations; Sweden, Finland and Estonia; Croatia, Turkey and Israel), but regional or continental groups were not suggested by the structure of the tree. To determine if populations that were poorly sampled contributed to the lack of phylogeographical structure, we made trees with a subset of well-sampled populations. These trees did not differ in general structure from that presented in Fig. 4.

For the AMOVA analysis, populations were grouped in different hierarchical arrangements to uncover groupings with the maximum value of Φ_{CT}. A wide array of groupings were tested (Table 3). Populations that were thought to be part of the same breeding population and were not significantly differentiated were always considered as a single interbreeding population (i.e. Portugal and Spain, France and Italy). The highest Φ_{CT} values were obtained when most Asian populations were considered independent and most populations from Canada and the USA were classified in the same group. European populations formed several groups. The maximum Φ_{CT} was 0.62 and Φ_{ST} was 0.69 (Table 3). The Φ_{CT} values obtained by grouping localities by continents or as North America and Eurasia showed that these groupings were clearly worse; variance among groups represented only 19% and 8%, respectively.

The number of migrants per generation (N_m) estimated using Φ_{ST} values from the pairwise comparison of
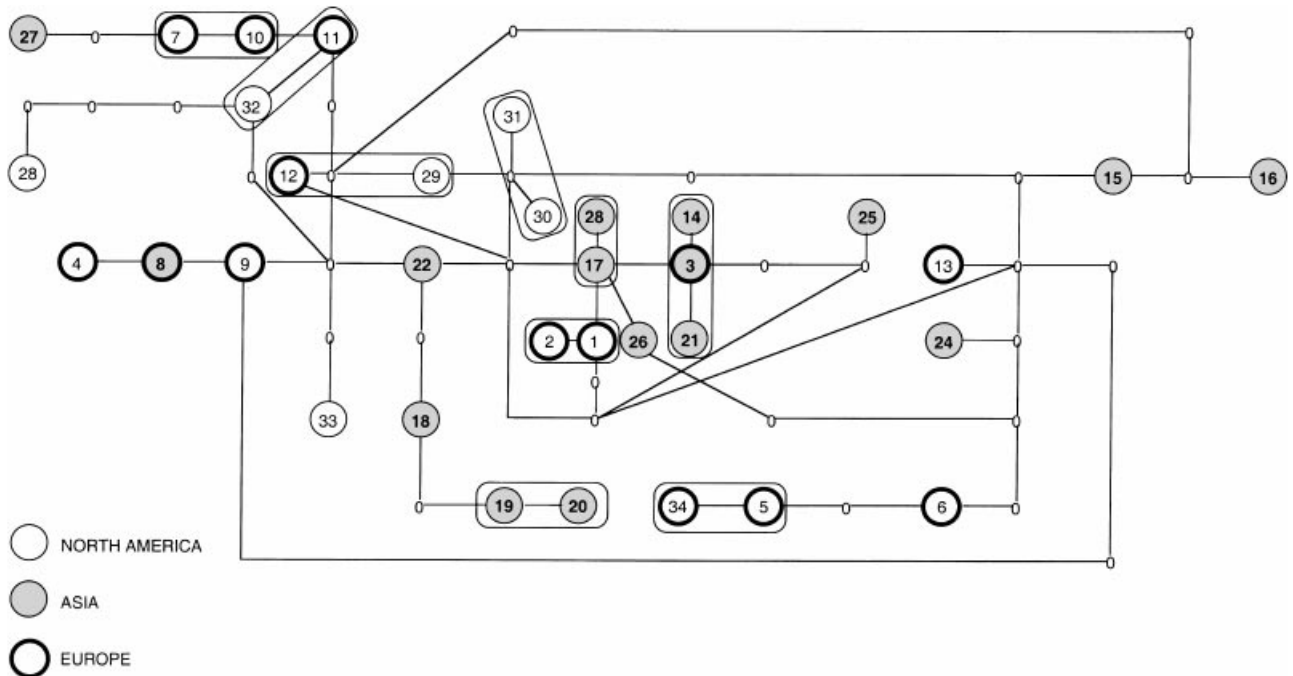
Fig. 3 Statistical parsimony cladogram (Templeton *et al*. 1992) of wolf haplotypes based on the number of substitutions and presence of indels between sequences. The numbers indicate the haplotypes as in Table 1. The continent where each haplotype was found is indicated. Only one-step clades are shown. Nesting at higher levels would make the graph unreadable.

Table 2 Nucleotide diversity (± standard deviation (SD)) for populations with five or more individuals (see Table 1)

	Nucleotide diversity (± SD)
Portugal	0.000907 (0.001286)
Spain	0.003806 (0.002983)
France	0
Italy	0
Croatia	0.007184 (0.005626)
Yugoslavia	0.027094 (0.016765)
Greece	0.016010 (0.010521)
Sweden	0.006423 (0.004544)
Israel	0
Saudi Arabia	0.020115 (0.012839)
Iran	0.001456 (0.001916)
Afghanistan	0.013915 (0.009135)
Mongolia	0.014778 (0.009607)
Mexico	0

populations suggested in the AMOVA analysis, did not show any significant relationship with distance (Fig. 5, Mantel's permutation test $P > 0.05$). For example, the number of migrants between the Iberian Peninsula (Spain + Portugal) and Italy plus France was 0.11 whereas it was 1.70 between the Iberian Peninsula and China. Similarly, the number of migrants between the Yukon and Greece +

Turkey was 4.00 whereas it was 0.09 between Yukon and Alaska. As most of these populations are currently isolated from each other, N_m values reflect past migration and methods using coalescence may be more appropriate. Maximum likelihood estimates of past migration per generation obtained using the program MIGRATE provided very similar results. We attributed these inconsistent values to small sample size, a high level of homoplasy and violations of the island model of migration (Whitlock & McCauley 1999).

Discussion

Divergence of grey wolves and coyotes and Pleistocene population cycles

The first grey wolves appeared in the Old World about 700 000 years ago (Kurtén 1968), and coyotes appeared in North America about 1 million years ago (Kurtén & Anderson 1980). We will consider, conservatively, that the *minimum* date for divergence of both lineages is 1 million years (see also Nowak (1979)). The sequence divergence between coyotes and grey wolves can be corrected for ancestral within-species polymorphism using the expression $pwc(net) = pwc - (pw + pc)/2$, where pwc is the sequence divergence (based on the HKY + Γ distance) between coyotes and wolves, and pw and pc the mean

Iran
Poland
Mexico
Israel
Turkey
Romania China Croatia
Spain
Italy
France Bulgaria Portugal
Mongolia
Russia Yukon
Finland
Estonia
Sweden
S. Arabia
Yugoslavia
Montana
Labrador Greece
NW Territories
Alberta India
Afghanistan
Minnesota
Alaska
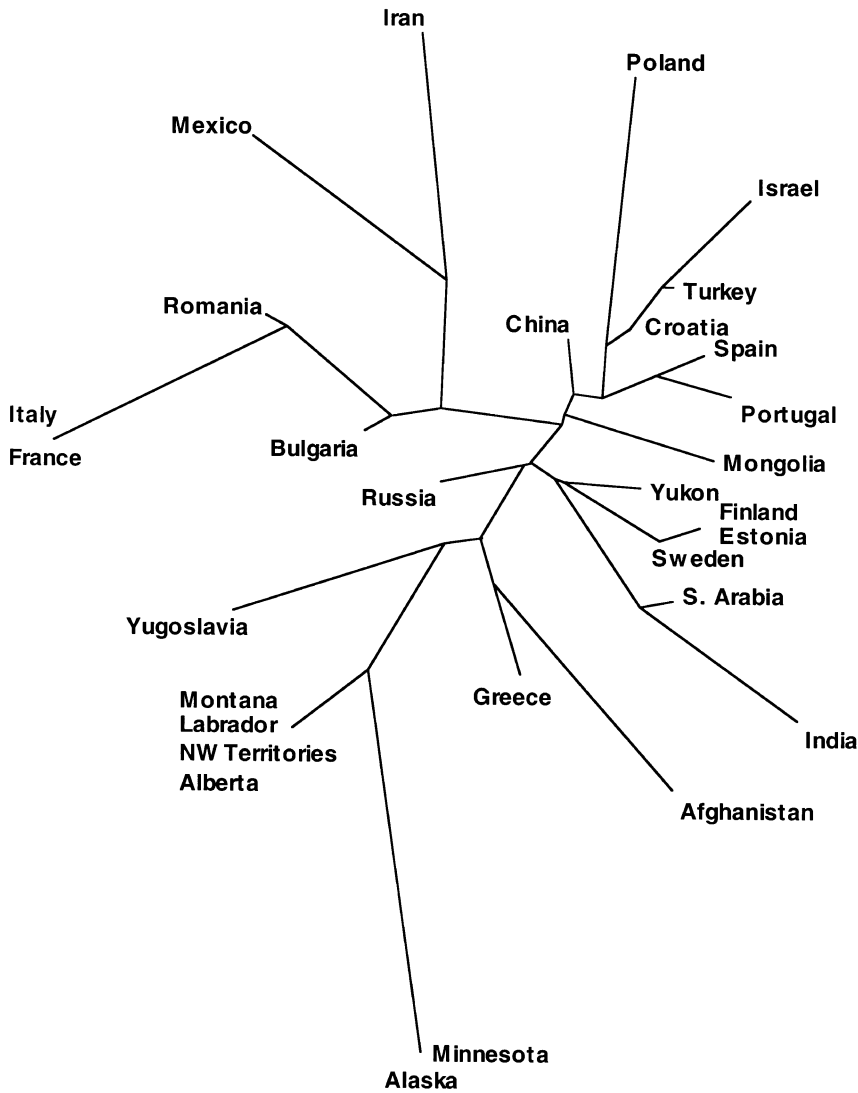
Fig. 4 Neighbour-joining tree of the populations based on the estimated number of net nucleotide substitutions between each pair of populations (Nei 1987). All nodes were supported in less than 50% of 100 bootstrap trees.

Table 3 Some examples of analysis of molecular variance (AMOVA). Fixation indices are indicated, as well as the percentage of the total variance that is explained by the grouping and its significance. For population names see Table 1

Groups	Φ_{SC}	Φ_{ST}	Φ_{CT}	% among groups	P
[Spain, Portugal] [France, Italy] [Romania, Bulg.] [Croatia] [Yugos.] [Poland, Estonia, Sweden, Finland, W Russia] [Greece, Turkey] [Israel] [Iran] [Afghan.] [India] [S Arabia] [China] [Mongol.] [Alaska] [Yukon] [NWT, Alb., Mont., Minn., Labra.] [Mexico]	0.185	0.689	0.619	61.89	< 0.01
[Eurasia] [America]	0.661	0.724	0.186	18.55	< 0.05
[Eurasia] [USA, Canada] [Mexico]	0.650	0.749	0.284	28.38	< 0.01
[Europe] [Asia] [USA, Canada] [Mexico]	0.660	0.689	0.086	8.55	0.09
[Spain, Portugal] [France, Italy] [Romania, Bulg.] [Croatia] [Yugos.] [Greece, Turkey] [Poland, Estonia, Sweden, Finland, W Russia] [Israel] [S Arabia] [Iran] [Afghan.] [India, China] [Mongol.] [Alaska] [Yukon, NWT, Alb., Mont., Minn., Labra.] [Mexico]	0.271	0.689	0.573	57.27	< 0.01
[Spain, Portugal] [France, Italy] [Romania, Bulg., Croatia, Yugos.] [Greece, Turkey] [Poland, Estonia] [Sweden, Finland] [Israel] [W Russia] [S Arabia] [Iran] [Afghan.] [India, China] [Mongol.] [Alaska] [Yukon, NWT, Alb., Mont., Minn., Labra.] [Mexico]	0.344	0.687	0.523	52.34	< 0.01
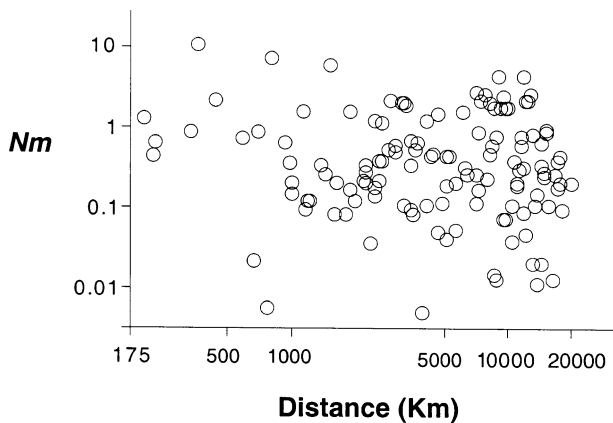
Fig. 5 Plot of the number of migrants per generation (N_m), estimated from Φ_{ST}-statistics, and the geographical distance between groups defined in the AMOVA analysis (see text). Some values of N_m approach infinity and are not included.

sequence divergence within wolves and within coyotes, respectively (Nei 1987; Avise & Walker 1998). The corrected sequence divergence between wolves and coyotes is 9.6%. Consequently, given a divergence rate of about 10% per million years and a mean sequence divergence in grey wolves of 2.9%, a coalescence of wolf haplotypes of about 290 000 years ago is implied. Similarly, the mean sequence divergence in coyotes of 4.2% implies coalescence about 420 000 years ago. Restriction site analysis of the whole mitochondrial genome found a similar value of 200 000–400 000 years ago as the coalescence time for grey wolves (Lehman *et al.* 1991; Wayne *et al.* 1992). However, the restriction site estimate of coalescence for coyotes is about 1 million years ago, as old as the divergence between wolves and coyotes (Lehman *et al.* 1991). Our results suggest that the coalescence is recent for both species.

This coalescence of wolf and coyote sequences is more recent than predicted from the current population size of both species. At equilibrium between drift and mutation, and assuming a low variance in family size, the expected coalescence time in generations is two times the effective number of females (Hartl & Clark 1989). For wolves and coyotes, the current census population size worldwide is of the order of hundreds of thousands and millions, respectively (Lehman *et al.* 1991). These numbers would predict a very old coalescence. The observed recent coalescence may be due to the effect of population fluctuations during Pleistocene glacial cycles on the harmonic mean of the effective population size. Historical fluctuations in population size cause the harmonic mean of the effective population size to be much smaller than the average census population size (Avise *et al.* 1984) and results in a more recent coalescence than predicted from census population size alone. Historical fluctuations in population size were probably common for grey wolves

and coyotes, as the Ice Ages must have imposed sharp reductions in the geographical range of both species. Population size reduction during glacial maxima was probably followed by expansion during interglacial times. Such serial cycles of population expansion and contraction throughout the Pleistocene would have dramatically decreased genetic variability below that predicted from interglacial population sizes (for example, in cheetahs, see Menotti-Raymond & O'Brien (1993)).

Recent demographic declines

Comparison of the $\hat{\theta}_T$ and $\hat{\theta}_F$ estimates provides insights into recent population history. While $\hat{\theta}_T$ reflects current diversity and demography, $\hat{\theta}_F$ uses genealogical information and thus reflects historical levels of variability (Kuhner *et al.* 1995). For both wolves and coyotes, $\hat{\theta}_F$ (0.744 and 0.373, respectively) is much larger than $\hat{\theta}_T$ (0.026 and 0.046, respectively). This indicates that both species have been much more diverse in the recent past and that wolves were more diverse historically than coyotes (see below).

To estimate historical population sizes in wolves, we assume a substitution rate of about 5×10^{-8} per nucleotide site per year for the control region (half of the sequence divergence between coyote and grey wolf, see above). Given a mean generation time of 3 years (Mech & Seal 1987), and a value of $\hat{\theta}_F$ equal to 0.744 in wolves, a historical effective population size of about 5 million breeding females is implied. Similarly, assuming a mean generation time of 2 years for coyotes (Bekoff & Wells 1986; Nowak 1991), a value of $\hat{\theta}_F$ equal to 0.373 implies an effective number of breeding females of 3.7 million. We suggest that the dramatic difference in the historical relative abundance of the two species reflects late Pleistocene changes in habitat continuity and area occupied by grey wolves. In the late Pleistocene wolves had a Holarctic distribution whereas the coyote was restricted to the central plains of North America (Mech 1970; Gier 1975; Bekoff & Wells 1986; Ginsberg & Macdonald 1990). Wolves are more dependent on forest habitats in many parts of their distribution (Mech 1970; Carbyn 1987; Voigt & Berg 1987) and during glacial times these habitats were severely fragmented and reduced in area (Hewitt 1996; Taberlet *et al.* 1998). In contrast, the plains and deserts of central North America were less sensitive to the fragmentation induced by Pleistocene climatic changes. Therefore, the genetic variability of coyotes may have been better preserved than that of wolves although their geographical distribution was less extensive. The distribution of pairwise differences (Fig. 4) supports this interpretation as wolves seem to have had a sharper population expansion than coyotes (Rogers & Harpending 1992; Rogers 1995).

The very recent decrease in genetic diversity suggested

by the comparison of $\hat{\theta}_T$ and $\hat{\theta}_F$ in coyotes and grey wolves may have different origins. In wolves, a substantial decrease in geographical range and population size has occurred during the last few centuries (Mech 1970; Carbyn 1987; Ginsberg & Macdonald 1990). Seton (1925) estimated that the prehistoric wolf population in North America was about 2 million individuals whereas the current population may be less than 60 000 individuals (Carbyn 1987). In contrast, coyotes have increased their geographical range several-fold in the last 100 years and they are now present throughout North America (Nowak 1979; Voigt & Berg 1987). However, this range expansion was too recent to have significantly increased the genetic variability of control region sequences. Rather, current levels of genetic variation may reflect a decrease in coyote numbers since the last glacial maximum, about 18 000 years ago, as grey wolves increased their distribution into postglacial forests (see Nowak (1979)). Here, as in Yellowstone National Park and elsewhere, grey wolves actively limit coyote numbers (Mech 1970; Robbins 1997).

The current population size of wolves and coyotes as indicated by $\hat{\theta}_T$ differs from that suggested by census estimates. The effective population size for females based on $\hat{\theta}_T$ is about 173 000 and 460 000 for wolves and coyotes, respectively. Assuming that 60% of adult female wolves breed, that the sex ratio is one and that about 50% of the population are adults (Mech 1970; Packard et al. 1983; in Wayne et al. 1992), the current census population size would be approximately 1 153 000 wolves. This number is much greater than the estimated worldwide wolf population size of less than 300 000 (Ginsberg & Macdonald 1990). The difference between the genetic and the census estimates probably reflects the recency of the population declines that is not yet well reflected in the loss of genetic variability worldwide (e.g. González et al. 1996). For coyotes, the total population size would be about 2.2 million assuming that 70% of adult female coyotes breed, that the sex ratio is one and that adults constitute 60% of the population (Connolly & Longhurst 1975; Lehman & Wayne 1991). This value is less than the estimate of 7 million individuals (1.75 million breeding females) based on census data (Lehman & Wayne 1991). As above, the higher census number in coyotes may be due to a population expansion too recent to have been recorded in the diversity of control region sequences.

The phylogeography and population structure of grey wolves

The statistical parsimony and population trees (Figs 3 and 4), AMOVA analysis (Table 3) and lack of differentiation by distance (Fig. 5), suggest an absence of large-scale geographical structure. A similar conclusion was reached by restriction site analysis of a smaller sample of wolves from fewer localities (Wayne et al. 1992). The multiple expansions and contractions to refugia that wolf populations have experienced during the Ice Ages, together with the changes in distribution of suitable habitats, may have contributed to the general lack of phylogeographical structure. Wolves are highly mobile predators, for which dispersal distances of several hundred kilometres are common and record movements over 1000 km have been recorded (Fritts 1983; Mech 1987; Mech et al. 1995). Consequently, during interglacials, wolf populations would rapidly expand into favourable habitats resulting in population admixture that would obscure past phylogeographical structure caused by Ice Age isolation. The effect of Ice Ages on the distribution of wolf haplotypes is apparent in the New World. Several episodes of migration have occurred across the Bering Land Bridge (Kurtén 1963, 1966). In North America, control region sequences of Yukon and Mexican grey wolves are divergent from those of other North American wolves (Fig. 1). The Mexican wolf sequence may be derived from an early invasion of wolves into North America (Wayne et al. 1992). Similarly, the presence of divergent sequences in Yukon may represent superimposed sequences from different migrations across the Bering Land Bridge. Because grey wolves are highly mobile, glaciers are only an ephemeral isolating barrier, and the admixture after glacial retreat would increase diversity in some populations and obscures past historical population structure.

A similar phenomenon, but over a more recent timescale, has occurred in North American coyotes. As discussed above, coyotes were previously restricted to the arid lands of the USA, and have vastly expanded their geographical range in the past 100 years to all of the continental USA and much of southern and central Canada. The genetic consequences of this expansion are apparent in a restriction fragment length polymorphism (RFLP) study of coyotes (Lehman & Wayne 1991). Divergent genotypes were found in several populations and genetic relationships between populations did not correspond to geography. For example, Californian coyotes had three times the genetic diversity of those from Minnesota, yet both are recently colonized states. The relationships of California genotypes varied as some grouped with those from Texas, others with those from Alaska (Lehman & Wayne 1991). A microsatellite analysis (Roy et al. 1994) has confirmed the lack of geographical associations among coyote populations. A similar rapid expansion from refugial populations during postglacial periods in wolves would have obliterated the previous geographical structure that might have existed and might account for the varying levels of genetic diversity. By contrast, population differentiation due to Ice Age isolation is still apparent in the brown bear *Ursus arctos* (Taberlet & Bouvet 1994; Kohn et al. 1995; Taberlet et al. 1995; Waits et al. 1998)

and black bear *U. americanus* (Wooding & Ward 1997), two species with lower levels of mobility.

The contraction of wolf populations in historical times, due to human persecution, has led to fragmentation and isolation. The majority of extant populations, especially in Eurasia, have unique haplotypes (Table 1). The expected time to fixation of a haplotype in a population is two times the effective number of females (Hartl & Clark 1989). Thus, given the assumptions above on the structure of wolf populations, a population of 220 individuals would be expected to be fixed for a single mitochondrial haplotype in two centuries. Some European wolf populations have reached population sizes clearly smaller than this, or have been fragmented into multiple populations of smaller size (Boitani 1982; Schröder & Promberger 1993; Ellegren *et al.* 1996). Thus, in the Old World, fragmentation and drift could contribute to the lack of correspondence between gene flow and distance through the random fixation of genotypes that were previously more widespread (Fig. 5; Wayne *et al.* 1992).

The statistical parsimony cladogram adds important resolution to our analysis. For example, restricted gene flow among regions is strongly supported by the observations that most one-step level clades include haplotypes from the same region (Fig. 3). Additionally, increasing the nesting level increases clade distances and tip clades have a smaller geographical range than interior clades (Templeton *et al.* 1995; Templeton 1998). Restricted gene flow can be explained by either recent habitat fragmentation as above or historical expansion followed by isolation by distance. The former seems better supported by historical evidence although a recent expansion is suggested in wolves by the unimodal distribution of pairwise differences (Fig. 2).

Units for conservation

Nearly all well-sampled population groupings were significantly differentiated with regard to genotype frequency and sequence divergence (Table 1). If nuclear data support this result, then each population might be considered as a separate management unit (Moritz 1994). The existence of significant morphological differences between wolf populations (Vilà 1993; Nowak 1996) supports their delineation as management units. However, our analysis shows that populations often contain divergent sequences. Additionally, a hierarchical geographical structure of populations was not evident. We interpret this as due to past episodes of isolation followed by admixture. Thus, the present-day fragmentation and differentiation of grey wolf populations should be viewed as a snapshot in a dynamic historical process that includes admixture. If wolf habitats were continuously distributed as in the past, many current management units might become less differentiated

and would not evolve into reciprocally monophyletic groups (evolutionarily significant units; Moritz 1994). From an evolutionary perspective, admixture was probably a common feature of the historical demography of the grey wolf, only recently interrupted by human disturbance. Thus, in general, individuals from neighbouring or closely related populations can justifiably be used as a source for re-introduction or population augmentation (e.g. for re-introductions in Yellowstone National Park, see Phillips & Smith (1996); for New Mexico, Hedrick *et al.* (1997)).

Final conclusions concerning the delineation of management units and the identification of source populations for re-introduction or augmentation require the analysis of nuclear markers (García-Moreno *et al.* 1996; Hedrick *et al.* 1997) and fitness-related phenotypic differences (Hedrick 1999). For example, the size of adult wolves in Arabia is one-third that of Alaskan wolves, and pelage patterns vary greatly among localities (Young & Goldman 1944; Vilà 1993). Similarly, in North America, analysis of nuclear loci in grey wolves found evidence for genetic differentiation among closely spaced populations not separated by geographical or habitat barriers (Roy *et al.* 1994; Forbes & Boyd 1997).

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